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Exhibit M

Benzodiazepines as Potent and Selective Bradykinin B_1 Antagonists

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Abstract: Antagonism of the bradykinin B_1 receptor was demonstrated to be a potential treatment for chronic pain and inflammation. Novel benzodiazepines were designed that display subnanomolar affinity for the bradykinin B_1 receptor (K_i = 0.59 nM) and high selectivity against the bradykinin B_2 receptor (K_i > 10 μM). In vivo efficacy, comparable to morphine, was demonstrated for lead compounds in a rodent hyperalgesia model.

Introduction. Bradykinin B_1 and B_2 receptors are G-protein-coupled receptors that function in pain and inflammation pathways.[1] The peptides, bradykinin and kallidin, act as the physiological agonists for the constitutively expressed bradykinin B_2 receptor to evoke acute pain immediately after tissue injury.[2] After physical trauma, bradykinin (BK) and kallidin are metabolized to [des-Arg^9]BK and [des-Arg^{10}]kallidin, which serve as the natural agonists for the bradykinin B_1 receptor.[3] While this receptor is not widely expressed in nondiseased states, it is induced upon injury and is believed to be active during persistent pain and inflammation. Recent studies suggest that the bradykinin B_1 receptor is constitutively expressed in the central nervous system of mice[4] and rats,[5] indicating a potentially central role for these receptors in addition to the accepted peripheral mode of action.

The therapeutic potential for a selective bradykinin B_1 antagonist has been supported by recent studies demonstrating that B_1 receptor knockout mice are outwardly normal and fertile while displaying a reduced sensitivity to various types of noxious, painful stimuli as well as inflammatory agents, such as carrageenan.[6] Additionally, known peptidic antagonists, [des-Arg^9, Leu^8]BK and [des-Arg^{10},Leu^9]kallidin, have shown promising results in chronic pain animal models.[7] Recently, a nociceptive spinal reflex assay in the rabbit demonstrated the analgesic efficacy of a different peptide B_1 antagonist,[8] B9858,[9] under conditions modeling chronic pain and inflammation. The lack of efficacy of B9858 in the absence of prior inflammation induced by complete Freund's adjuvant (CFA) implies that a selective bradykinin B_1 antagonist should restrict its activity to sites of prior insult, thereby limiting undesirable side effects. While these known peptidic antagonists have helped validate bradykinin B_1 receptors as a target, they do not have the physical properties or pharmacokinetic characteristics required of an orally active pharmaceutical. Herein, we present the development of selective, small-molecule, non-peptidic bradykinin B_1 antagonists with demonstrated in vivo efficacy against inflammatory pain.

Screening of our in-house sample collection identified benzodiazepine 1 as an antagonist of the human bradykinin B_1 receptor (K_i = 28.0 nM; fluorescence imaging plate reader (FLIPR) IC_{50} = 164 nM) with excellent selectivity over the bradykinin B_2 receptor (K_i > 10 μM).[10] Related compounds were therefore synthesized.

Chemistry. The compounds appearing in Table 1 were prepared according to Scheme 1. The benzodiazepine cores, if not commercially available, were prepared from 2-aminobenzonitrile and the requisite Grignard reagent.[11] The isocyanates were prepared in a straightforward manner from 4-fluoronitrobenzene and commercially available amines via a three-step sequence. This entailed nucleophilic aromatic fluoride displacement followed by a Pd/C catalyzed hydrogenation of the nitro group to form the aniline, which was converted in situ to the corresponding isocyanate with triphosgene.[12]

Biological Results and Discussion. K_i values (nM) were determined radiometrically using the appropriate radioligand and Chinese hamster ovary (CHO) cells stably expressing the human bradykinin B_1, rat B_1, or human B_2 receptor. In vitro functional activity was assessed in standard FLIPR experiments (IC_{50}, nM) employing functionally active human B_1 or rat B_1 receptors. Full details for the above experiments are described in the Supporting Information.

The optimization of lead compound 1 initially focused on increasing the binding affinity to the human bradykinin B_1 receptor. Cognizant of the high frequency with which basic moieties appear in ligands for G-protein-coupled receptors, we believed that the dimethylamino terminus was important for binding. Consistent with this hypothesis, replacement of the dimethylamino moiety with a hydrogen provided a compound (2) devoid of bradykinin B_1 receptor binding. Since removal of the basic side chain was clearly deleterious, modification of the dimethylamino group was pursued. These efforts resulted in the introduction of a piperidine terminus that more than doubled the affinity and functional activity over the initial lead compound (comparing a racemic mixture of 3 and 4 to the racemate 1). Separation of enantiomers[13] demonstrated that the (*R*) enantiomer (3) was 2 orders of magnitude more potent than the (*S*) enantiomer (4). Assignment of the (*R*) configuration as the higher affinity enantiomer was based in

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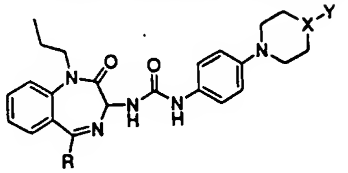
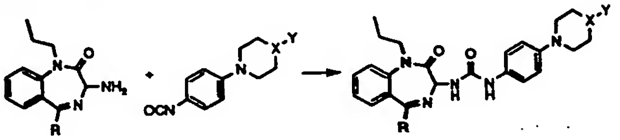
Table 1. Bradykinin B_1 Receptor Binding Affinities and Rat Pharmacokinetics for the Benzodiazepines



compd[a]	R	X	Y	human K_i [b] (nM)	human FLIPR IC_{50} [b] (nM)	rat K_i [b] (nM)	rat FLIPR IC_{50} [b] (nM)	rat %*F*, $t_{1/2}$ (h)
(±)-**1**	*c*-hexyl	CH	*N*,*N*-dimethylamino	28.0	164			
(±)-**2**	*c*-hexyl	CH	H	>10000				
(*R*)-**3**	*c*-hexyl	CH	1-piperidinyl	5.73	24.7	18.3	59.0	10(±3), 3.2 (±0.7)
(*S*)-**4**	*c*-hexyl	CH	1-piperidinyl	1023	2200			
(*R*)-**5**	phenethyl	CH	1-piperidinyl	1.37	4.83	1.97	7.25	4.9(±4.5), 2.4(±0.3)
(*R*)-**6**	*p*-tolyl	CH	1-piperidinyl	13.4	64.5	17.0	27.0	32(±14), 4.3(±0.9)
(*R*)-**7**	*c*-hexyl	N	4-pyridinyl	1.33	8.00		24.2	2.5(±0.4), 2.3(±0.6)
(±)-**8**	*c*-hexyl	N	3-pyridinyl	85.0				
(±)-**9**	*c*-hexyl	N	2-pyridinyl	97.5				
(±)-**10**	*c*-hexyl	N	phenyl	>10000				
(±)-**11**	phenethyl	CH	4-pyridinyl	55.0				
(*R*)-**12**	phenethyl	N	4-pyridinyl	0.59	1.90	0.92	2.80	3.4(±0.5), 2.7(±0.5)

[a] All compounds were >95% pure by HPLC and characterized by ^{1}H NMR and HRMS. [b] Values represent the numerical average of at least two experiments. Interassay variability was ±10% for the binding assays and ±25% for the FLIPR experiments.

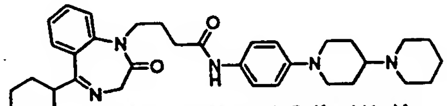
Scheme 1



analogy to C-5 phenyl, *N*-1-methylbenzodiazepine analogues of known configuration (not shown).

Further enhancement of the inhibitory activity of these compounds was achieved through modification of the benzodiazepine C-5 substituent. In general, alkyl groups extending less distance than cyclohexyl (methyl, phenyl, *tert*-butyl) at this location afforded substantially decreased affinity (data not shown). However, elongating the C-5 substituent from *c*-hexyl to phenethyl (**5**) enhanced affinity by 4-fold at the human receptor and 9-fold at the rat bradykinin B_1 receptor (compare to **3**). Correspondingly, the bradykinin B_1 functional activity of **5** was improved 5-fold and 8-fold at the human and rat receptors, respectively. Supporting the hypothesis that extended alkyl groups were preferred at this location, the addition of just a single methyl group at the 4-position on a phenyl ring (**6**) restored potency to within roughly 2-fold of **3**.

Another breakthrough in affinity occurred when the piperidinyl–piperidinyl portion of **3** was replaced with a 4-pyridinylpiperazinyl motif (compound **7**). This modification imparted a 4-fold increase in affinity relative to its predecessor **3**. However, the position of the pyridine nitrogen proved to be critical. Movement of this nitrogen to either the 3- or the 2-position (racemic **8** and **9**, respectively) precipitated a drop in affinity of over 30-fold relative to **7** (single enantiomer). As with compound **2**, deletion of the basic pyridine nitrogen to provide **10** abrogated affinity for the bradykinin B_1 receptor. The remaining aniline nitrogen in **10** is either not sufficiently basic to bind to the receptor or not in the proper location to bind effectively. Also detrimental to binding, albeit less so, was the replacement of the distal piperazine nitrogen (X = CH), resulting in compound **11**. This 50-fold drop in affinity (comparing racemic **11** to the single enantiomer **12**) could be attributed to a combination of effects. The reduced basicity of the pyridinyl nitrogen concomitant with a significant change in conformation represents the most likely causes for the decreased binding affinity of **11**.



13, human bradykinin B_1 K_i = 141 nM

Figure 1. Relocation of the basic side chain from the C-3 position to the end of the N-1 alkyl group provides a new class of achiral compounds that retain significant bradykinin B_1 affinity.

Combining the potency-enhancing modifications described above into a single antagonist provided compound **12**. Consistent with the concept of additive structure–activity relationships (SAR), this compound possessed subnanomolar affinity for both the human and rat bradykinin B_1 receptors while also displaying excellent functional activity.

Figure 1 depicts an extensive structural modification of antagonist **3** that still allows for high affinity at the bradykinin B_1 receptor. In this achiral molecule **13**, the entire basic side chain has been relocated to the end of the N-1 alkyl group (of optimized chain length) and attached by an amide. Surprisingly, only a 25-fold reduction in affinity is observed, relative to **3**. Examination of models reveals that, because of the flexibility of the *n*-alkyl linker at the N-1 position, the basic functionality still has the ability to access a similar region of space compared to previous analogues where the attachment occurs at the C-3 position. However, the possibility of a second basic-binding pocket on the receptor cannot be ruled out. An alternative explanation for this compound's B_1 receptor affinity could be that the basic side chain has not altered its location in the binding site. Instead, the benzodiazepine portion may have moved into a different position. Regardless of which explanation is correct, this example and the preceding modifications highlight the importance of having a properly configured basic side chain.[14]

In Vivo Characterization. We were encouraged to find that the first compound tested in rat, **4**, displayed 10% oral bioavailability with an intravenous (iv) half-life of 3.2 h. Unfortunately, with the introduction of the potency-enhancing phenethyl group at the C-5 position in **5**, there was a 2-fold reduction in bioavailability and a drop in half-life (Table 1). However, while introduction of a *p*-tolyl group at this C-5 position decreased affinity only slightly, there was an improvement in the rat pharmacokinetics for **6** to the highest levels observed: 32% oral bioavailability with a 4.3 h iv half-life. Modification of the basic side chain by introduction of the other potency enhancer, the 4-pyridinylpiperazine found in **7**, reduced bioavailability 4-fold relative to **3**. Following this downward trend, the bioavailability for **12**, now containing both potency-enhancing modifications, was in line with the values obtained for its closest analogues (**5** and **7**). Although the bioavailabilities were not acceptable for oral dosing, compounds **3** and **12** were of sufficient potency to be tested in a rat pain model.

The model chosen was a carrageenan-induced hyperalgesia assay in the rat.[15] In brief, hyperalgesia is induced in the hind paw of a Sprague–Dawley rat by the intraplantar injection of carrageenan. The ability of a compound to inhibit this hyperalgesia is then measured by the latency of the inflamed hind paw to withdraw from the application of increasing pressure, where suppressed hyperalgesia is expressed in terms of a percentage relative to untreated animals. Compound **3** (rat K_i = 18.3 nM; FLIPR IC_{50} = 59.0 nM) administered via intraperitoneal (ip) injection was ineffective at the 1 mg/kg level. However, when dosed at 3 and 10 mg/kg, 27 ± 15% and 80 ±10% inhibition of hyperalgesia (n = 8/group) was observed in a dose-dependent fashion. As anticipated, the 20-fold more potent analogue **12** (rat K_i = 0.92 nM; FLIPR IC_{50} = 2.80 nM) performed even better. At a dose of 1 mg/kg (ip), 27 ± 22% inhibition was observed, and at a dose of 3 mg/kg (ip), 89 ± 14% of the carrageenan-induced hyperalgesia (n = 6/group) was suppressed. By comparison, morphine dosed (ip) at 0.3, 1.0, and 3.0 mg/kg resulted in 24 ± 19%, 74 ± 24%, and 108 ± 20% inhibition (n = 6/group), respectively, with the highest dose showing early signs of hypoalgesia. Importantly, the IC_{50} values for **12** against rodent opioid receptors were all greater than 1 μM.[16] Thus, the in vivo efficacy of benzodiazepine **12** and morphine was of comparable strength despite their different target receptors. While the in vivo antihyperalgesic activities of **3** and **12** are consistent with bradykinin B_1 antagonism, this assay is not specific for the B_1 mechanism, and therefore, contribution of potential unknown off-target activities cannot be ruled out. It is considered, however, that selective bradykinin B_1 antagonists will be efficacious against chronic pain and inflammation, similar to morphine, but without the deleterious side effects associated with the opiates.

In conclusion, novel bradykinin B_1 receptor antagonists have been identified, with the most potent members demonstrating subnanomolar binding affinity and low-nanomolar functional activity. These compounds have enabled the demonstration of in vivo efficacy comparable to morphine for the suppression of hyperalgesia. The above observations support the therapeutic potential for a bradykinin B_1 antagonist in the treatment of chronic inflammatory pain.

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Supporting Information Available: Spectral data (^{1}H NMR and HPLC), HRMS data of new compounds, a representative description for the preparation of **12**, experimental details for the bradykinin B_1/B_2 binding and FLIPR assays, and the protocol for rat pharmacokinetics. This material is available free of charge via the Internet at http://pubs.acs.org.

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(12) A more detailed description for the preparation of these compounds can be found in the Supporting Information. Serving as a representative example, the preparation of compound **12** is elaborated.

(13) Enantiomers were separated and analyzed for enantiomeric excess (in all cases >98%) on L-leucine Pirkle type columns (preparative and analytical) using chloroform and methanol (0.1% diethylamine) as eluants.

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(16) Additionally, **12** shows high selectivity (>100×) against a panel of assays (Panlabs, MDS Pharma Services, Bothell, WA) representing 156 enzymes, receptor, and transporters (including cannabinoid, dopamine, histamine, opiate, and $GABA_A$ benzodiazepine receptors and transporters) with the following exceptions: human tachykinin $NK_2 > 30\times$, human leukotriene $B_4 > 60\times$, human muscarinic $M_1 > 70\times$, and human muscarinic $M_4 > 90\times$.

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